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INFLUENCE OF EXPLANTS TYPE AND PLANT GROWTH REGULATORS ON *IN VITRO* MULTIPLE SHOOTS REGENERATION OF *VANILLA PLANIFOLIA*

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ABSTRACT

The frequency of shoot bud regeneration was influenced by the type of explant, genotype and concentrations of plant growth regulators. An efficient in vitro multiplication protocol was developed through excised shoot tip and nodal segment of Vanilla planifolia and was studied with an aim to select best explant and effect of different plant growth regulators (PGRs) alone or in combination on multiple shoots production from different explants of V. planifolia. Explants viz. shoot tips and nodal segments of vanilla were cultured on MS medium augmented with different concentrations of BAP/Kinetin/NAA. Among the various concentrations tested, MS medium supplemented with 3.0 mg/L BAP and 1.0 mg/L NAA was found to be the best for maximum shoot bud differentiation. Maximum number of multiple shoots per explant (11.60 shoots/explant) was obtained from nodal segments.

KEYWORDS: BAP, Kinetin, Multiple Shoot, NAA, Nodal Segment, Shoot Tip & Vanilla

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Original Article

INTRODUCTION

Vanilla planifolia Andrews (vanilla) is climbing vine belongs to the orchid family (Orchidaceae). *Vanilla* comprises more than 110 species. *Vanilla planifolia* is also known as *V. fragrans* Salisb. This species is native to Central America. In present days, this orchid is cultivated in tropical regions including India, Madagaskar and Indonesia and has become commonly distributed. Apart from *V. planifolia*, some other *Vanilla* species (*V. tahitensis* and *V. pompona*) are also cultivated but *V. planifolia* is regarded as the as the best natural source of vanillin ($C_3H_8O_3$) which is an economically important flavouring substance. Extracted vanillin is as a flavouring ingredient in preparation of cakes, chocolate and also some beverages. Apart from using vanillin in edible preparations, it is used in cosmetics, perfume industry. Due to its antioxidant properties, it is widely used in pharmaceuticals also. Therefore the market demand of vanillin is increasing day by day in a significant manner[1].

Usually, Vanilla seeds do not germinate. Therefore maximum propagation of Vanilla is done through stem cuttings. This method of propagation is not only labour intensive and time consuming, but also it retards the growth and development of the mother plant. Thus it has become difficult to produce Vanilla through stem cutting to satisfy the market demand. Tissue culture has tremendous potential in this context and could be useful in overcoming the above mentioned limitations. For effective multiplication and supply of this orchid vine, the potential of tissue culture must be exploited for large scale production. This study concerns the influence of different plant growth regulators on *in vitro* shoot multiplication of *V. planifolia* from nodal explants and shoots tips and some attempts have been made to increase the number of adventitious shoots *in vitro* culture.

MATERIALS AND METHODS

Plant Material and Explant Preparation

The experiment was conducted at commercial plant tissue culture laboratory, Assam Agricultural University, Jorhat, with the objective to evaluate the effect of different sterilants on explants in vanilla for *in vitro* culture. The explants were taken from the Horticultural orchard of Assam Agricultural University, Jorhat. In the laboratory, the Explants were cut into 2 cm long and were rinsed with clean tape water and soaked in fungicide solution (carbendazim 'Derosal' 0.10%) for one hour before transfer to laminar flow. Then inside the laminar flow, a 30 seconds treatment was done with 70% alcohol which is followed by an one hour treatment with a mixture of 100% (v/v) hypochlorite (3.85% sodium hypochlorite) and few drops of Tween 20. The next step is followed by a treatment of 50% (v/v) hypochlorite for 30 minutes. In order to remove the remaining hypocholrite before initiation, the explants were trimmed and rinsed with sterile distilled water.

Culture Conditions and Media for Shoot Proliferation

Properly sterilized excised shoot tips and nodal segment (containing axillary buds) explants were inoculated singly in glass culture bottles containing 20 ml of culture media containing MS basal salts supplemented with 30 g/l sucrose, vitamins; glycine 2 g/l, pyridoxine 0.5 g/l, Nicotinic acid 0.5 g/l, Thiamine 0.1 g/l and Myo inositol at 0.1 g/l. The media was also supplemented with different concentrations of BAP, Kinetin and NAA as shown in (Table I) and solidified with 8 g/l of agar. The pH of the media was adjusted to 5.8 before autoclaving the media at 121°C and 15 psi for 20 min. The medium was then cooled at room temperature before use.

Table 1: Composition of Modified MS Media Tested for Multiple Shoot Production

Media	Basal Media	BAP (MG/L)	Kinetin (MG/L)	NAA (MG/L)
VPS_1	MS	-	-	-
VPS_2	MS	1.0	-	0.2
VPS_3	MS	1.5	-	0.5
VPS_4	MS	2.0	-	1.0
VPS_5	MS	2.5	-	1.0
VPS_6	MS	3.0	-	1.0
VPS_7	MS	-	1.0	0.2
VPS_8	MS	-	1.5	0.5
VPS_9	MS	-	2.0	1.0
VPS_{10}	MS	-	2.5	1.0
VPS_{11}	MS	-	3.0	1.0

VPS = *Vanilla planifolia* Shooting Media, MS = Murashige and Skoog (MS) medium

Inoculation of Plant Materials

The shoot tip and nodal explants of about 1 cm long were directly inoculated singly to each culture bottle containing MS semi solid basal medium supplemented with different concentrations of auxins (NAA) and cytokinins (BAP and Kn) and covered with plastic cap. After that the caps were sealed with parafilm. Each treatment was repeated thrice. MS medium without growth regulators served as the control.

Transfer to Growth Chamber

For the growth and development of the cultures, a controlled environment of 25±2ºC temperature and 16-hour photoperiod of 200 lux light intensity was maintained in the growth room where the cultured bottle are transferred and

allowed to grow.

Sub-Culturing

The explants were inoculated for 30 days during the initial stages and the produced clumps of shoots were separated and divided into single shoots for periodic sub-culture on every 30 days in the same media for multiplication. During the process of sub-cultures, shoots were cute into single pieces trimming any blackish or brownish tissue. A similar fresh medium was used for each piece. Micro shoots produced during *in vitro* proliferation were also separated and sub-cultured in fresh medium. Sub-culturing was done for a period 7 weeks and response of explants were recorded in terms of growth, number of shoots, shoot length and multiplication rate. This experimental data was collected and recorded at the end of the 7^{th} week from the subculture.

Statistical Analysis

The experiments were set up in a completely randomized design. Data were analysed by analysis of variance to detect significant differences among the mean of treatments using Duncan's multiple range test (DMRT) at 5% significance level.

RESULTS

In the present experiment, the initial shoots derived from both the explants were cultured on MS medium supplemented with different combinations of BAP, Kinetin and NAA. After seven weeks in all types of media, the response, proliferation and multiplication of *Vanilla planifolia* explants were observed and recorded.

No. of Explants Responded for Multiple Shooting

The percentage of both the explants responding to multiple shoot induction was found to be highest (90.00%) on VPS_6 medium supplemented with MS+BAP 3.0mg/L+NAA 1.0 mg/L whereas lowest response was found on VPS_7 medium (15.00% nodal explant and 20.00% shoot tip explant) (Table II & III) supplemented with MS+ Kinetin 1.0 mg/L+NAA 0.2 mg/L, while single shoot was not regenerated in control (Table III).

Table 2: Effect of Different Modified MS Media for multiple Shoot Production from Established Nodal Explants of Vanilla Planifolia Andrews

Medium[a]	No. of Nodal Explants Responded for Multiple Shooting	Percentage of Nodal Explants Responded for Multiple Shooting	Days Required for Multiple Shoot Initiation	After 7 Weeks of Culture		
				No. of Shoots Produced per Explant	Shoot Length (cm)	No. of Leaves per Shoot
VPS_1	0	0 (0.00)	0.00	0.00	0.00	0.00
VPS_2	9^{d}	45 (42.13)	33.91^{d}	2.88^{e}	2.34^{de}	3.10^{cd}
VPS_3	10^{cd}	50 (45.00)	29.67^{de}	3.78^{e}	3.00^{cd}	2.28^{d}
VPS_4	13^{bc}	65 (53.73)	26.97^{ef}	8.56^{bc}	4.90^{a}	5.90^{a}
VPS_5	16^{ab}	80 (63.44)	26.67^{ef}	10.12^{ab}	4.24^{ab}	4.80^{b}
VPS_6	18^{a}	90 (71.57)	22.40^{f}	11.60^{a}	3.60^{bc}	4.00^{bc}
VPS_7	3^{e}	15 (22.79)	45.60^{a}	2.40^{e}	1.88^{e}	2.90^{d}
VPS_8	4^{e}	20 (26.56)	39.91^{bc}	3.15^{e}	2.20^{de}	2.80^{d}
VPS_9	11^{cd}	55 (47.87)	42.71^{ab}	6.20^{d}	2.90^{cd}	3.10^{cd}
VPS_{10}	14^{bc}	70 (53.79)	32.33^{d}	7.00^{cd}	3.10^{c}	3.30^{c}
VPS_{11}	14^{bc}	70 (53.79)	30.93^{de}	8.16^{cd}	2.78^{cd}	2.90^{d}
SEd±	**1.938**		**2.305**	**0.860**	**0.393**	**0.344**
CD at 5 %	**4.129**		**4.911**	**1.833**	**0.838**	**0.733**

*No. of explants inoculated = 20,

Figures in parentheses are arcsine transformation values.

[a]For media composition see Table I.

Means within columns separated by Duncan's multiple range test P = 0.05

Means followed by same letter shown in superscript (s) are not significantly different.

Table 3: Effect of Different Modified MS Media for Multiple Shoot Production from Established Shoot Tip Explants of Vanilla Planifolia Andrews

Medium[a]	No. of Shoot Tip Explants Responded for Multiple Shooting	Percentage of Shoot Tip Explants Responded for Multiple Shooting	Days Required for Multiple Shoot Initiation	After 7 Weeks of Culture		
				No. of Shoots Produced per Explant	Shoot Length (cm)	No. of Leaves per Shoot
VPS_1	0.00	0.00 (0.00)	0.00	0.00	0.00	0
VPS_2	3.67^{def}	36.70 (37.27)	37.2^{c}	2.83^{bc}	2.53^{cd}	1.13^{e}
VPS_3	5.00^{cde}	50.00(45.00)	34.63^{cde}	3.23^{ab}	3.57^{bc}	2.03^{d}
VPS_4	6.67^{bc}	66.70 (54.74)	32.25^{def}	3.40^{ab}	5.09^{a}	4.40^{a}
VPS_5	8.00^{b}	80.00 (63.44)	31.67^{ef}	3.47^{ab}	4.85^{a}	3.47^{a}
VPS_6	9.00^{a}	90.00 (71.57)	28.07^{f}	3.70^{a}	3.97^{b}	2.98^{b}
VPS_7	2.00^{f}	20.00(26.57)	48.27^{a}	2.40^{c}	2.54^{cd}	2.05^{d}
VPS_8	3.00^{ef}	30.00(33.21)	43.78^{b}	2.80^{bc}	2.20^{d}	2.82^{bc}
VPS_9	5.67^{cd}	56.70 (48.83)	46.72^{ab}	2.87^{bc}	3.02^{cd}	2.60^{c}
VPS_{10}	7.00^{abc}	70.00 (56.79)	36.33^{cd}	2.93^{bc}	3.72^{b}	2.20^{d}
VPS_{11}	7.00^{abc}	70.00 (56.79)	34.93^{cde}	3.20^{ab}	2.78^{cd}	2.17^{d}
SEd±	**0.985**		**1.801**	**0.337**	**0.285**	**0.165603**
CD at 5 %	**2.098**		**3.836**	**0.718**	**0.607**	**0.3527**

*No. of shoot tip explants inoculated =10, Figures in parentheses are arcsine transformation values.

[a]For media composition see Table I. Means within columns separated by Duncan's multiple range test P = 0.05

Means followed by same letter shown in s superscript(s) are not significantly different.

Number of Shoots Per Explant

Maximum number of shoots proliferation per explant was found on VPS_6 medium supplemented with MS+BAP 3.0mg/L+NAA 1.0 mg/L) (11.60 in nodal explant and 3.70 in shoot tip explant) after 7 weeks of culture (Plate 1). Nodal segment showed the best performance than shoot tip. VPS_7 medium recorded lowest number of shoots per explant (2.44 in nodal explant and 2.40 in shoot tip explant)).

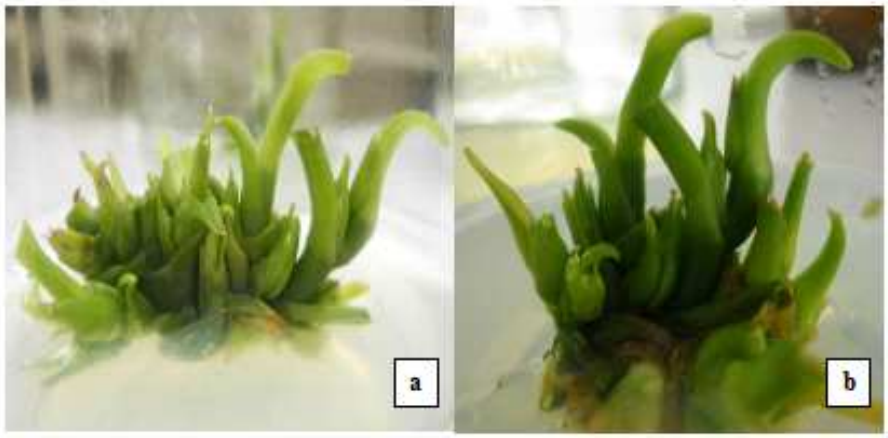

Plate 1: Multiple Shoot Production and Growth from Established of (a) Nodal Segment (b) Shoot Tip Explant of Vanilla Planifolia Andrews in VPS_6 Medium

Shoot Length

Shoot length also influenced by cultivars and different combinations of BAP, Kinetin and NAA. Longest shoot was found %) on VPS_4 medium supplemented with MS+ 2.0 mg/L BAP and 1.0 mg/L NAA in both the explants, but the shoot-tip explant produced longer shoot (5.09 cm) compared to nodal explant (4.90) after 7 weeks of culture (Table I & III, Plate 2). The lowest shoot length was recorded on VPS_7 medium by nodal segment (1.88 cm) and by shoot tip explant (2.20 cm) on VPS_8 medium (Table I).

Plate 2: Elongated Shoots of Vanilla Planifolia Andrews after Division of Multiple Shoots on VPS_4 Medium

Days to Single Shoot development

Early single shoot was developed at VPS_6 medium supplemented with MS+BAP 3.0mg/L+NAA 1.0 mg/L) (22.40 days by nodal segment and 28.07 days by shoot-tip explant) (Table II & III). The VPS_7 medium took highest days or longer duration (45.60 days for nodal explant & 48.27 days for shoot-tip explant) to initiate multiple shoots.

Number of Leaves Per Plantlet

Among all the treatments, on VPS_4 medium supplemented with MS+ 2.0 mg/L BAP and 1.0 mg/L NAA showed maximum number of leaves in both the explants. The nodal explant produced 5.90 leaves per plantlet in VPS_6 medium

while shoot-tip explant produced 4.40 leaves per plantlet at the same medium (Table II & III). While the lowest (2.28) number of leaves was recorded on medium VPS_3 in case of nodal segment and the lowest (1.13) number of leaves was recorded on medium VPS_2 in case of shoot tip explant.

DISSCUSSIONS

The treatment with 3.0 mg/L BAP in combination with 1.0 mg/L NAA resulted in the highest proliferation (highest shoots/explant, minimum days requirement for shoot proliferation) as compared to control and other combinations. However, the length was found to be decreased on the above medium. This may be due to the fact that suppression of apical dominance leads to the production of more number of multiple shoots and reduced shoot length. This was in accordance with the results of Murashige (1974) wherein for axillary bud proliferation; cytokinin has been utilized to overcome apical dominance of shoot and enhances branching of lateral buds from leaf axils. Hormonal control of organogenesis as reported by Skoog and Miller (1957) states that a high cytokinin to auxin ratio favours shoot differentiation. According to Wickson and Thimann (1958), cytokinins could release the lateral buds from apical dominance. Presence of cytokinins stimulate the growth and elongation of the dormant buds of vegetative apex. Similar report was obtained by George and Ravishankar (1997), when multiple shoots were produced by axillary bud explants using semi-solid MS medium supplemented with 6-benzyladenine (BAP) at 2.0 mg/L + NAA at 1.0 mg/L and obtained an average of 5.70 shoots per axillary bud explants. Lisha (2003) was also obtained multiplication rate of 4.11 ± 0.99 shoots per explant by after culturing nodes on SH media supplemented with 3.0 mg/L BAP for 75 days.

Action of BAP is the principal cause of shoot proliferation in tissue culture. Optimum dose of BAP enhances axillary and multiple branching and thus multiple shoots are achieved. Furthermore, the optimum cytokinin level is also genotype dependent (Anandana *et al*., 2000). Similarly increase in NAA concentration beyond an optimal concentration induces callus formation and thus retards the axillary bud sprouting. It is well established fact that the optimal ratio of cytokinin : auxin causes the dormant meristematic zone existing in the nodal segment or shoot tip to initiate shoot organogenesis.

The mean number of leaves per shoot and shoot length were maximum in medium supplemented with 2.0 mg/L BAP and 1.0 mg/L NAA. The elongation in stem is not due to increased formation of nodes and internodes but as the result of rapid elongation of internodal cells, which is due to both cell divisions followed by cell elongation. The present studies showed that BAP was more effective than Kinetin in enhancing shoot multiplication. These results are in accordance with the work of Girija *et al.* (1999), Mohapatra and Rout (2004), Nagesh (2008) and Tan *et al.* (2011), who found that the proliferation rate of explants cultured on the medium supplemented with kinetin was generally lower than BAP.

CONCLUSIONS

Stimulation of multiple shoot formation was achieved by culturing the established explants of vanilla in the shoot multiplication media (MS medium supplemented with different concentrations of BAP, Kinetin and NAA). For both the explants MS medium supplemented with 3.0 mg/L BAP and 1.0 mg/L NAA was found to be the best in terms of minimum days required for multiple shoot initiation and maximum number of multiple shoots per explant. Whereas, MS medium supplemented with 2.0 mg/L BAP and 1.0 mg/L NAA was found to be the best in terms of shoot length and no. of leaves per explant. But for the commercial plantlet regeneration, Nodal explant was found better than shoot tip for shoot regeneration.

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